#13

PATENT 4/6/99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR THE DIAGNOSIS AND TREATMENT OF CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

8/19/98
DATE

SIGNATURE

DECLARATION OF DR. GARY L. CLAYMAN UNDER 37 C.F.R. § 1.131

Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

I, Gary L. Clayman, D.D.S., M.D., declare that:

1. I am a U.S. citizen residing at 6353 Westchester Street, Houston, Texas. I am Associate Professor of Surgery and Deputy Chairman of the Department of Head and Neck Surgery at the University of Texas M.D. Anderson Cancer Center. A copy of my curriculum vitae outlining my education and research training is attached (Exhibit A).

2. I am the inventor of the above-captioned application and a portion of my research has been sponsored by Introgen Therapeutics, Inc., a company that has licensed this technology.

3. I am a co-author of Clayman *et al.*, published in *Cancer Research* (Exhibit B), along with Drs. Adel K. El-Naggar, Jack A. Roth, Wei-Wei Zhang, Helmuth Goepfert, Dorothy L. Taylor, and Ta-Jen Liu. I also am co-author of Liu *et al.*, published in *Cancer Research* (Exhibit C), along with Drs. Liu, El-Naggar, Taylor, Timothy J. McDonnell, Kim D. Steck, and Mary Wang.

4. Drs. El-Naggar, Roth, Zhang, Goepfert, Taylor, Liu, McDonnell, Steck and Wang, the non-inventor co-authors of this paper, did not contribute to the conception of the present invention of using Adp53 for the treatment of head and neck cancer. Each of these individuals acted under the supervision and direction of myself in generating the results reported in these papers, or as a reviewer of the manuscripts prior to publication.

5. Dr. Adel K. El-Naggar performed the pathologic analysis in Exhibit B and the fluorescent analysis in Exhibit C.

6. Dr. Jack A. Roth provided the Adp53 vector in Exhibits B and C.

7. Dr. Wei-Wei Zhang developed the Adp53 vector in Exhibit B.

8. Dr. Helmuth Goepfert reviewed the Exhibit B manuscript prior to publication.

9. Dr. Dorothy L. Taylor developed and maintained the head and neck cancer cell lines, and assisted in the experiments described in Exhibits B and C.

10. Dr. Ta-Jen Liu performed the *in vitro* propagation of the Adp53 vector in Exhibit B and performed the DNA fragmentation analysis in Exhibit C.

11. Dr. Timothy J. McDonnell reviewed the Exhibit C manuscript prior to publication.

12. Dr. Kim D. Steck performed the technical fluorescent sorting studies in Exhibit C.

13. Dr. Mary Wang performed infection assays and viral propagation in Exhibit C.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made herein on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the U.S. Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/18/98
Date

Gary L. Clayman, D.D.S., M.D.

CURRICULUM VITAE

NAME: Gary L. Clayman, D.D.S., M.D., F.A.C.S.

Present Title and Affiliation:

Deputy Chairman
Associate Professor of Surgery and Associate Surgeon, and Director of Research
Department of Head and Neck Surgery
The University of Texas M.D. Anderson Cancer Center

Faculty, Graduate School of Biomedical Sciences
The University of Texas at Houston
Health Science Center

Adjunct Associate Professor
Baylor College of Medicine
Department of Otolaryngology and Communicative Sciences

Adjunct Associate Professor
The University of Texas Dental Branch
Department of Oral Biology and Oral Medicine

Birth Date and Place:

November 21, 1957
Cleveland, Ohio

Citizenship: United States

Home Address:

6353 Westchester Street
Houston, Texas 77005
Phone: (713) 661-8541

Office Address:

U. T. M.D. Anderson Cancer Center
1515 Holcombe Blvd - 69
Room R10.2248
Houston, Texas 77030
Phone: (713) 792-8837
Fax: (713) 794-4662
E-mail: gclayman@notes.mdacc.tmc.edu

Marital Status: Married: Judith K. Wolf, M.D.

Licensures - Active:

Medical:	Texas	H-5856 (1989)	
	Minnesota	0312594 (1987)	inactive
Dental:	Ohio	17114	inactive

Certification:

American Board of Otolaryngology
Head and Neck Surgery
April 7, 1992

Joint Council for Approval of Advanced Training in Head and Neck Oncologic Surgery-1992

Fellow of the American College of Surgeons
October 1995

Education:

Case Western Reserve University,
Cleveland, Ohio
1976-1978
Degree Granted: BS, Biology

Case Western Reserve University
School of Dentistry
Cleveland, Ohio
1978-1982
Degree Granted: DDS

Northeastern Ohio Universities
College of Medicine
Rootstown, Ohio
1982-1986
Degree Granted: MD

University of Minnesota
Minneapolis, Minnesota
1987-1991
Degree Granted: MS, Immunobiology

Postgraduate Training:

Clinical Fellow, Department of Head and Neck Surgery, UT M.D. Anderson Cancer Center
Houston, Texas
1991- 1993

Postgraduate Training (cont'd):

Research Fellow, Department of Head and Neck Surgery, UT M.D. Anderson Cancer Center
Houston, Texas
1989-1990

Resident, University of Minnesota
Department of Otolaryngology
Minneapolis, Minnesota
1987-1991

Intern, Hennepin County Medical Center
Department of General Surgery
Minneapolis, Minnesota
1986-1987

Subintern, Case Western Reserve University Hospital, Department of Oral and Maxillofacial Surgery
Cleveland, Ohio
1981-1982

Specialty Boards:

Otolaryngology/Head and Neck Surgery
1991 - Written exam successfully completed

1992 - Oral exam successfully completed

Academic and Professional Appointments:

UT M.D. Anderson Cancer Center
Deputy Chairman and Associate Professor of Surgery and Associate Surgeon, and Director of Research Department of Head and Neck Surgery
1997 - present

UT M.D. Anderson Cancer Center
Associate Professor of Surgery and Associate Surgeon, and Director of Research
Department of Head and Neck Surgery
1996 - 1997

Academic and Professional Appointments (cont'd):

UT M.D. Anderson Cancer Center
Associate Professor of Tumor Biology and Associate Biologist
Department of Tumor Biology
1996 - present

Baylor College of Medicine
Adjunct Associate Professor
Department of Otorhinolaryngology and Communicative Sciences
January 1, 1996 - present

The University of Texas at Houston
Dental Branch Adjunct Associate Professor
1996-present

UT M.D. Anderson Cancer Center
Assistant Professor of Surgery and Director of Research
Department of Head and Neck Surgery
1993 - 1996

American Academy of Otolaryngology
Head and Neck Surgery, Inc.
Chair of the Head and Neck Surgery and Oncology Committee
October 1996-1999

The Graduate School of Biomedical Sciences
Associate Faculty
The University of Texas at Houston Health Science Center
1993 - present

The University of Texas at Houston
Dental Branch
Adjunct Assistant Professor
June 1993 - 1996

Academic and Professional Appointments (cont'd):

UT. M.D. Anderson Cancer Center
Junior Faculty Associate
Department of Head and Neck Surgery
1992-1993

UT. M.D. Anderson Cancer Center
Clinical Fellowship
Department of Head and Neck Surgery
1991-1992

UT. M.D. Anderson Cancer Center
Research Fellowship
Department of Head and Neck Surgery
1989-1990

University of Minnesota Hospitals and Clinics Residency Department of Otolaryngology
1987-1991

Hennepin County Medical Center
University of Minnesota,
Internship,
Department of Surgery
1986-1987

Self Employed Private Practice
General Dentistry
1982-1986

Case Western Reserve University
School of Dentistry, Department of Oral and Maxillofacial Surgery
1981-1982

Administrative Responsibilities:

Deputy Chairman
Department of Head and Neck Surgery
1997 – present

Administrative Responsibilities (cont'd):

Director of Research
Department of Head and Neck Surgery
1994 - present

Program Director
Multidisciplinary Program in Head and Neck Oncology Research
1996 - present

Division of Surgery
Promotions Committee
1996 - present

IRB (Institutional Surveillance Committee)
1995 - present

Institutional Research Program Development and Direction Committee
1995 - present

Faculty Senate Research Issues Committee
(Institutional) 1996 - present

Graduate Medical Education Committee
1995 -1997

Local, State, National, International Committees:

Chairman, Head and Neck Oncology Committee
American Association of Otolaryngology and Head and Neck Surgery, Inc.
1997 - present

National Institute of Dental Research Advisory Committee 1997

Local, State, National, International Committees: (cont'd)

Endocrine Surgery Subcommittee
American Academy of
Otolaryngology - Head and Neck
Surgery
1996 - present

Head and Neck Oncology
Committee
American Association of
Otolaryngology and
Head and Neck Surgery, Inc.
1995 - 1996

Radiation Therapy Oncology Group
(RTOG) Subcommittee of Head and
Neck Surgery
1995 - present

RTOG Head and Neck Oncology
Research Committee
1995 - present

SPF Advisory Committee, The
American Cancer Society
1996 - present

American Society for Head and
Neck Surgery
Research Committee
1996 - present

Society of Head and Neck Surgeons
Publication Committee
1996 - present

Society of Head and Neck Surgeons
Research Committee
1996 - present

Society of Head and Neck Surgeons
Resident Award Committee
1996 - present

Editorial and Review Board:

PRS Research Committee,
UT M.D. Anderson Cancer Center
Sept 1996 - present

Clinical Affairs Committee, UT
M. D. Anderson Cancer Center
1996 - present

Current Drugs Committee,
UT M.D. Anderson Cancer Center
1997
Laryngoscope
Editorial Board
1996 - present

Cancer
Reviewer
1995 - present

Nature Medicine
Reviewer
1997 - present

Cancer Research
Reviewer
1995 - present

Journal of Head and Neck Surgery
Reviewer
1991-present

Laryngoscope
Reviewer
1991 - present

Archives of Otolaryngology/Head
and Neck Surgery
Reviewer
1993 - present

Texas Medicine
Reviewer
1993 - present

Editorial and Review Board (cont'd):

American Cancer Society
Immunobiology Section

Ad Hoc Reviewer
1991-present

Journal of Clinical Immunology
Ad Hoc Reviewer
1991-present

New England Journal of Medicine
Ad Hoc Reviewer
1991 - present

Honors and Awards:

American Cancer Society Career Development Award
1993 - 1996

American Society for Head and Neck Surgery, Scholastic Award in Basic Science Research
1992

Allergy and Immunology
Janssen Case History Contest
1991

University Cancer Foundation
Award of
UT M.D. Anderson Cancer Center
1991-1992

The American Cancer Society,
Texas Division
Oncology Fellowship Award
1991

The American College of Surgeons
Residents
Research Award
1990

Honors and Awards (cont'd):

Society of Head and Neck Surgeons
Resident/Fellow Clinical Award
1990

Outstanding Surgical Intern of the Year, Hennepin County Medical Center
1987

Joseph A. Keller Award
1985

American College of Surgeons
Medical

Student Fellowship Award
1985

Arthur W. Shagrin Scholarship of Northeastern
Ohio Universities College of Medicine
1984-85

Charles F. Block Foundation
Scholarship Award 1983

Case Western Reserve University
School of Dentistry Undergraduate
Research Award
1982

Alpha Omega Dental Honor Society
1981

Phi Beta Kappa Honor Society
1981

Summa Cum Laude, Case Western Reserve University 1980

Miller, Ratner, Safran Foundation
Scholarship 1977-82

Honors and Awards (cont'd):

Jacee Wives Scholarship
1976

Case Western Reserve University
Pre-professional Scholarship
Program
1976-82

Society Memberships:

American Medical Association

American College of Surgeons -
Fellow

American Society of Head and Neck
Surgeons
Fellow

Society of Head and Neck Surgery -
Fellow

American Academy of
Otolaryngology-
Head and Neck Surgery - Fellow

Society of University
Otolaryngologists

American Association for Cancer
Research

American Society of Clinical
Oncology

American Academy of Facial Plastic
and Gary L. Clayman, D.D.S., M.D.

Reconstructive Surgery - Member
Texas Medical Association

Minnesota Medical Association

Harris County Medical Society

Ramsey County Medical Society

Society Memberships (cont'd):

Hennepin County Medical Society

American Dental Association

The Houston Society of
Otolaryngology/
Head and Neck Surgeons

American Radium Society

**Grant Support for
the Past Five Years:**

P05 DE11906-01 Oral Cancer Research Center
Novel Diagnosis and Therapy of Early Oral
Cancers Project #4 Apoptosis Inducing Gene
Therapy for Oral Premalignancy
PI: Gary L. Clayman
Percent Effort: 15%
$3,722,559
September, 1, 1997-August 30, 2001

R29 DE11689-01A1 Developing Molecular
Therapy in Head and Neck Cancer
PI: Gary L. Clayman
Percent Effort: 50%
$517,999
October 1, 1996 - June 30, 2001

P01 Ca 68233
Biology of Non-Melanoma Skin Cancer
Growth and Progression: Core C
PI: Gary L. Clayman
Percent Effort: 10%
$473,945
July 01, 1996 - June 31, 2001

Sponsored Research Agreement-Introgen
Therapeutics, Inc. Modification of Tumor
Suppressor Gene Expression in Head and
Neck

Grant Support for the Past Five Years (cont'd):

Squamous Cell Carcinoma (HNSCC) with Adenovirus Vector Expression Wild-Type p53
PI: Gary L. Clayman
Percent Effort 10%
$2,000,000
October 1, 1995 - June 30, 1998
U01 Ca68089-01

Biochemoprevention for Advanced Premalignant Lesions in the Upper Aerodigestive Tract:
PI: W.K. Hong
Percent Effort: 10%
$492,551
September 14, 1995 – Aug. 31, 1998

T32 Ca60374-01A2
Training of Academic Head and Neck Surgical Oncologists
PI: Helmuth Goepfert, M.D./Ruben Lotan, Ph.D.
Percent Effort:5%
$356,832
August 1, 1995 - May 31, 2000

Physicians Referral Service
Developing Gene Therapy
$35,000
July 1, 1994 - June 30, 1996

American Cancer Society
Career Development Award
$90,000
July 1, 1993-June 30, 1996

Bibliography:

Published Articles:

1. **Clayman GL**, Goldberg J. The incidence of forceps delivery among patients with TMJ problems. *Journal Cranio-Mandibular Practice;* 1:46-53, 1983.

2. **Clayman GL**, Marentette L. Complex odontoma of the maxillary sinus. *Otolaryngology/Head and Neck Surgery;* 100:189-192, 1989.

3. **Clayman GL**, Adams G. Permanent tracheostomy with cervical lipectomy. *Laryngoscope;* 100:422-424, 1990.

4. **Clayman GL**, Savage HE, Ainsley N, Liu FJ, Schantz SP. Serologic determinants of survival in patients with squamous cell carcinoma of the head and neck. *American Journal of Surgery;* 160:434-438, 1990.

5. Schantz SP, **Clayman GL**, Liu FJ, Lavedan P, Taylor DL, Pellegrino C, Savage HE. The in-vivo biologic effect of interleukin-2 and interferon-alpha on natural immunity in head and neck cancer patients. *Archives of Otolaryngology;* 116:1302-1308, 1990.

6. Schantz SP, **Clayman GL**, Dimery I, Moric R. Combination interleukin-2 and interferon-alpha in head and neck cancer. *Cancer Bulletin;* 43:133-138, 1991.

7. **Clayman GL**, Adams G. Modifications of the mandibular swing for preservation of occlusion and function. *Head and Neck Surgery;* 13:102-106, 1991.

8. **Clayman GL**, Liu FJ, Taylor DL, Savage HE, Lavedan P, Buchsbaum RM, Trujillo JM, Schantz SP. Immunomodulation of the induction phase of lymphokine-activated killer activity by acute phase proteins. *Otolaryngology/Head and Neck Surgery;* 105:234-239, 1991.

9. **Clayman GL**, Adams GL, Paugh DR, Koopmann CF Jr. Intracranial complications of paranasal sinusitis: a combined institutional review see comments. *Laryngoscope;* 101(3):234-239, 1991.

10. **Clayman GL**, Adams GL, Szachowicz EH, Dehner LP, Gorlin RJ. Oropharyngeal desmoid fibromatosis, congenital glaucoma and cataracts, and a calvarial defect: A new syndrome. *Otolaryngology:Head and Neck Surgery;* 107(1) 109-114, 1992.

11. **Clayman GL**, Lui FJ, Savage HE, Taylor DL, Lavedan P, Buchsbaum RM, Pellegrino C, Trujillo JM, Young G, Schantz SP. Acute-phase proteins in patients with head and neck cancer treated with interleukin-2/interferon alfa. *Archives of Otolaryngology-Head and Neck Surgery;* 118:41-48, 1992.

Published Articles (cont'd):

12. **Clayman GL**, Young G, Grimm E, Taylor DL, Schantz SP. Immunologic profile of a patient with dyskeratosis congenita. *Immunology & Allergy Practice;* 14(4):17-24, 1992.

13. Byers RM, **Clayman GL**, Guillamondegui OM, Peters LJ, Goepfert H. Resection of advanced cervical metastasis prior to definitive radiotherapy for primary squamous carcinoma of the upper aerodigestive tract. *Head and Neck Surgery;* 14(2):133-138, 1992.

14. **Clayman GL**, Young G, Taylor DL, Savage HE, Lavedan P, Schantz SP. Detection of regulatory factors of lymphokine-activated killer cell activity in head and neck cancer patients treated with interleukin-2 and interferon alpha. *Annals of Otology, Rhinology & Laryngology;* 101(11):909-915, 1992.

15. **Clayman GL**. Acute-phase proteins and interleukin 6 serum level in head and neck cancer. [Letter To The Editor] *Archives Otolaryngology Head and Neck Surgery;* 118:1366-1367, 1992.

16. Schantz SP, Dimery I, Lippman SM, **Clayman GL**, Pellegrino C, Morice R. A Phase II study of interleukin-2 and interferon-alpha in head and neck cancer. *Investigational New Drugs;* 10:217-23, 1992.

17. Lydiatt DL, Savage HE, **Clayman GL**, Liu FJ, Sample D, Schantz SP. Serologic determinants of survival in patients with head and neck cancer: validating a clinical prediction model. *The Laryngoscope;* 103:13-16, 1993.

18. **Clayman GL**, Taylor DL, Liu FJ, Lavedan P, Savage HE, Schantz SP. Serum and acute phase protein modulation of the effector phase of lymphokine-activated killer cells. *Laryngoscope;* 103(3):299-307, 1993.

19. **Clayman GL**, Cohen JI, Adams GL. Neoplastic seeding of squamous cell carcinoma of the oropharynx. *Head and Neck* 15(3)245-248, 1993.

20. **Clayman GL**, Wang SW, Nicolson GL, El-Naggar A, Mazar A, Henkins J, Blasi F, Goepfert H, Boyd DD. Regulation of urokinase-type plasminogen activator expression in squamous-cell carcinoma of the oral cavity. *International Journal of Cancer;* 54:73-80, 1993.

21. Stern SJ, Goepfert H, **Clayman GL**, Byers R, Wolf P. Orbital preservation in maxillectomy. *Otolaryngology and Head and Neck Surgery;* 109(1):111-115, 1993.

22. Stern SJ, Goepfert H, **Clayman GL**, Byers R, Ang KK, EL-Naggar AK, Wolf P. Squamous cell carcinoma of the maxillary sinus. *Archives of Otolaryngology-Head and Neck Surgery;* 119(9):964-969, 1993

Published Articles (cont'd):

23. Juarez J, **Clayman GL**, Nakajima M, Tanabe KK, Saya H, Nicolson GL, Boyd D. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. *International Journal of Cancer;* 55:10-18, 1993.

24. **Clayman GL**, Raad II, Hankins PD, Weber RS. Bacteriologic profile of surgical infection after antibiotic prophylaxis. *Head and Neck;* 15:526-531, 1993.

25. Duvic M, Nelson DC, Annarella M, Cho M, Esgleyes-Ribot T, Remenyik E, Ulmer R, Rapini RP, Sacks PG, **Clayman GL**, Davies PJA, Thacher S. Keratinocyte transglutaminase expression varies in squamous cell carcinomas. *The Journal of Investigative Dermatology;* 102(4):462-469, 1994.

26. Liu T-J, Zhang W-W, Taylor DL, Roth JA, Goepfert H, **Clayman GL**. Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. *Cancer Research;* 54(13):3662-3667, 1994.

27. **Clayman GL**, Stewart MG, Weber RS, El-Naggar AK, Grimm EA. HPV in laryngeal and hypopharyngeal carcinomas: relationship to survival. *Archives of Otolaryngology/Head and Neck Surgery;* 120:743-748, 1994.

28. **Clayman GL**, El-Naggar AK, Roth JA, Zhang W-W, Goepfert H, Taylor DL, Liu T-J. In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Research;* 55:1-6, 1995.

29. Janot F, El-Naggar AK, Morrison RS, Liu T-J, Taylor DL, **Clayman GL**. Expression of basic fibroblast growth factor in squamous cell carcinoma of the head and neck is associated with degree of histologic differentiation. *International Journal of Cancer ;* 64:1-7, 1995.

30. **Clayman GL**, Weber RS, Guillamondequi O, Byers RM, Wolf PF, Frankenthaler RA, Morrison WH, Garden AS, Hong WK, Goepfert H. Laryngeal preservation for advanced laryngeal and hypopharyngeal cancers. *Archives of Otolaryngology-Head and Neck Surgery;* 121:219-223, 1995.

31. Lengyel E, Gum R, Juarez J, **Clayman GL**, Seiki M, Sato H, Boyd D. Induction of M_r 92,000 type IV collagenase expression in a squamous cell carcinoma cell line by fibroblasts. *Cancer Research;* 55:963-967, 1995.

32. **Clayman GL**, Trapnell BC, Mittereder N, Liu T-J, Eicher S, Zhang S, and Shillitoe EJ. Transduction of normal and malignant oral epithelium by an adenovirus vector: The effect of dose and treatment time on transduction efficiency and tissue penetration. *Cancer Gene Therapy;* 2:105-111, 1995.

Published Articles (cont'd):

33. **Clayman GL**, Chamberlain RM, Lee JJ, Lippman SM, Hong WK. Screening at a health fair to identify subjects for an oral leukoplakia chemoprevention trial. *Journal of Cancer Education;* 10:88-90, 1995.

34. Liu TJ, El-Naggar AK, McDonnell TJ, Steck KD, Wang M, Taylor DL, **Clayman GL.** Apoptosis induction mediated by wild-type p53 adenovirus gene transfer in squamous cell carcinoma of the head and neck. *Cancer Research;* 55:3117-3122, 1995.

35. Kanjilal S, Strom SS, **Clayman GL**, Weber RS, El-Nagger AK, Kapur V, Cummings KK, Hill LA, Spitz MR, Kripke ML, and Ananthaswamy HN. p53 mutations in non-melanoma skin cancer of the head and neck: molecular evidence for field cancerization. *Cancer Research;* 55:3604-3609, 1995.

36. **Clayman GL.** Basic Science Reviews: Gene therapy for head and neck cancer. *Head and Neck;* 17:535-541, 1995.

37. Beckhardt RN, Kiyokawa N, Xi L, Liu T-J, Hung M-C, Zhang H-Z, El-Naggar AK, **Clayman GL.** HER-2neu oncogene characterization in head and neck squamous cell carcinoma. *Archives of Otolaryngology-Head and Neck Surgery;* 121:1265-1270, 1995.

38. **Clayman GL**, DeMonte F, Jaffee DM, Schusterman MA, Weber RS, Miller MJ, Goepfert H. Outcome and complications of extended cranial base resection requiring microvascular free tissue transfer. *Archives of Otolaryngology-Head and Neck Surgery;* 121:1253-1257, 1995.

39. Trizna Z, **Clayman GL**, Spitz MR, Briggs KL, Goepfert H. Glutathiones transferase genotypes as risk factors for head and neck cancer. *American Journal of Surgery;* 170:499-501, 1995.

40. **Clayman GL**, Liu T-J, Overholt M, Mobley SR, Wang M, Janot F, Goepfert H. Gene therapy for head and neck cancer. Comparing the tumor suppressor gene p53 and a cell cycle regulator WAF1/C1P1 (p21). *Archives of Otolaryngology-Head and Neck Surgery,* 122:489-493, 1996.

41. Fueyo J, Gomez-Manzano C, Yung WKA, **Clayman GL**, Liu T-J, Bruner J, LevinVA, and Kyritsis AP. Adenovirus-mediated p16/CDKN2 gene transfer induces growth arrest and modifies the transformed phenotype of glioma cells. *Oncogene;* 12:103-110, 1996.

42. El-Naggar, AK, Lovell M, Killary AM, **Clayman GL**, Batsakis JG. A mucoepidermoid carcinoma of minor salivary gland with t(11;19) (q21;p13.1) as the only karyotypic abnormality. *Cancer Genet Cytogenet,* 87:29-33, 1996.

Published Articles (cont'd):

43. Kim SK, Fan Y, Papadimitrakopoulou V, **Clayman GL**, Hittleman WN, Hong WK, Lotan R, Mao L. DCPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Research 56:2519-2521, 1996.*

44. Garden AS, Morrison WH, **Clayman GL**, Ang KK, Peters LJ. Early squamous cell carcinoma of the hypopharynx: outcomes of treatment with radiation alone to the primary disease. *Head and Neck 18 17-322, 1996.*

45. Califano J, van der Riet P, Westra W, Nawroz H, **Clayman G**, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, Sidransky D. Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Research 56:2488-2492, 1996.*

46. Gillenwater A, Xu X-C, El-Naggar AK, **Clayman GL**, Lotan R. Expression of galectins in head and neck squamous cell carcinoma. *Head and Neck 18:422-432, 1996.*

47. Mobley SR, **Clayman GL**,. The promise of gene therapy in head and neck cancer. *Current Opinion in Otolaryngology & Head and Neck Surgery* 4:82-87, 1996.

48. Liu TJ, El-Naggar AK, McDonnell TJ, Steck KD, Wang M, Taylor DL, **Clayman GL.** Apoptosis induction mediated by wild-type p53 adenovirus gene transfer in squamous cell carcinoma of the head and neck. *Cancer Research;* 55:3117-3122, 1995.

49. Papadimitrakopoulou V, Izzo J, Lippman SM, Lee JS, Fan, YH, **Clayman GL**, Ro JY, Hittleman WN, Lotan R, Hong WK, Mao L. Frequent inactivation of $p16^{ink4a}$ in oral premalignant lesions. *Oncogene* 14;1799-1803, 1997.

50. Xia, W, Lau, Y-K, Zhang H-Z, Liu A-R, Kiyokawa, N, **Clayman, GL**, Katz RL, Hung M-C. Strong Correlation Between c-erbB-2 Overexpression and Overall Survival of Patients with Oral Squamous Cell Carcinoma *Clinical Cancer Research.* Vol 3; 3-9, 1997.

51. Geara, FB, Glisson, BS, Sanguineti G, Tucker, SL, Garden, AS, Ang, KK, Lippman SM, **Clayman GL**, Goepfert H, Peters, LP, Hong WK. Induction Chemotherapy Followed by Radiotherapy Versus Radiotherapy Alone in Advanced Nasopharyngeal Carcinoma: Results of a Matched Cohort Study. *Cancer* 79; 1279-1286, 1997.

52. Hunt KK, Deng Jiong, Liu T-J, Wilson-Heiner M, Swisher SG, **Clayman GL**, Hung M-C. Adenovirus-Mediated Overexpression of the Transcription Factor E2F-1 Induces Apoptosis in Human Breast and Ovarian Carcinoma Cell Lines and Does Not Require p53. *Cancer Res* 57,pp 477-4726, Nov 1997.

Published Articles (cont'd):

53. El-Naggar AK, Lai S, **Clayman GL,** Lee, J-K J, Luna MA, Goepfert H, Batsakis J. Methylation, a Major Mechanism of p16/CDKN2 Gene Inactivation in Head and Neck Squamous Carcinoma. *Am J Pathol,* Vol 151, Dec 1997.

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55. Mobley, Steve, Liu, T.J., Hudson, J. Michael., **Clayman, GL.** In Vitro Growth Suppression by Adenoviral Transduction of p21 and p16 in squamous cell carcinoma of the head and neck; a research model for combination gene therapy. *Archives of Otolaryngology-Head and Neck Surgery p88-92, Jan 98.*

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Preliminary Results of a Phase I clinical study of Ad-p53 in Head and Neck Squamous Cell Carcinoma RPR Gencell Partners Meeting. Chantilly, Virginia. November 19, 1996.

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[CANCER RESEARCH 55, 1–6, January 1, 1995]

Advances in Brief

In Vivo Molecular Therapy with *p53* Adenovirus for Microscopic Residual Head and Neck Squamous Carcinoma[1]

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Abstract

Developing gene therapy strategies may allow contemporary medicine to reassess its management of solid malignancies. We have demonstrated previously that the wild-type *p53* adenovirus (Ad5CMV-p53) suppressed the growth of established tumors of the head and neck. In this paper we develop a microscopic residual model which mimics the postsurgical environment of head and neck cancer patients with advanced disease. Using this squamous cell carcinoma of the head and neck model, we prevented the establishment of tumors in nude mice in which tumor cells had been s.c. implanted by transiently introducing exogenous wild-type *p53* via an adenoviral vector 2 days following tumor cell implantation. These effects were vector dose dependent but independent on the endogenous wild-type or mutated *p53* status of the cells. Importantly, karyotypically normal and nontumorigenic fibroblast cell lines are inert to the *p53* adenovirus treatment. These results pave the ground work for further development of molecular therapy for head and neck cancer and other solid malignancies.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects upon speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients, approximately 50%, has remained unchanged for the nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Recurrences among these patients remain predominantly local and regional; approximately only 10% of patients die of distant metastasis alone (2, 3). In patients with SCCHN, the pathological findings of extracapsular invasion, neurotropism, and microscopic residual disease necessitate adjunctive therapy and predict aggressive local-regional disease. Moreover, these factors can usually be predicted prior to surgical intervention.

In head and neck cancer, direct gene transfer to microscopic residual carcinoma may not be technically difficult. When the primary tumor is removed, the tumor milieu is readily accessible for molecular therapy and is the most likely pathway of lymphatic spread when the regional lymphatic dissection is performed. Therefore, novel means of addressing assumed microscopic residual disease using direct transfer of genes that encode toxic products, specific tumor suppressor genes, or genes that induce products that specifically promote tumor cell death and spare nonmalignant cells may provide desperately needed improvement in local-regional control among these patients and thus be an important approach to cessating these malignancies. In addition, several other solid malignancies possess the same dilemma, and therefore the model of SCCHN may provide insight into cancers of several other organ systems.

We believe that promising new therapies for SCCHN are interventions at the molecular level, and adenovirus-mediated gene transfer is our clinical method of choice for such intervention. Adenoviruses have a known tropism for the epithelium of the aerodigestive tract and are linked only to minor disease in humans (4). Moreover, in contrast to retroviruses, they are capable of transferring genes to nonproliferating cells, which appears preferable because of the heterogeneity of cell cycling within the tumor microenvironment (5). Finally, the transient nature of gene expression after adenoviral gene delivery allows selection of a molecular intervention that will provide the desired outcome (tumor cell death in cancer) without long-term integration of the recombinant molecular therapy into bystander cells and the potential ramifications thereof.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138, Tu-177, MDA 686-LN, and MDA 886 were all established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, and had been characterized previously (6, 7). These cells were grown in DMEM (DMEM/Ham's F-12) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection, Cell Growth Assay, and Western Blot Analysis. All the procedures have been described previously (8). Cell growth assays were all performed in triplicate.

***In Vivo* Transduction with β-Galactosidase Adenovirus.** X-Gal staining of tissue specimens were performed on ornithine carbamyl transferase [Tissue Tek O. C. T. Compound® (Miles, Elkhart, IN)] frozen tissue sections to determine transduction efficiency. Eight-μm-thick specimens were washed in cold PBS and fixed in 0.5% glutaraldehyde at room temperature for 5 min. Slides were then washed twice with 4°C PBS and incubated for 4 h in X-Gal solution [1.3 mM $MgCl_2$, 15 mM NaCl, 44 mM Hepes buffer (pH 7.4); 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 2% X-Gal in *N,N*-dimethyformamide]. Slides were counterstained with H & E.

Immunohistochemical Analysis. Formalin-fixed paraffin-embedded *in vivo* animal experimental tissues were cut at 4–5 μm, dried at 60°C, deparaffinized, and hydrated with distilled water. Sections were then treated with 0.5% saponin in distilled water and rinsed in several changes of distilled water; endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, followed by rinsing in several changes of distilled water. Sections were microwave-irradiated in distilled water for 3 min using a Sharp Model R9H81 microwave oven operating at a frequency of 2450 MHz at 700 W. After cooling, sections were washed in several changes of distilled water and placed in PBS; immunochemical studies were performed by using the avidin-biotin-peroxidase complex method of Hsu *et al.* (9) in the following manner: sections were blocked with normal horse serum and incubated overnight at 4°C with

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[2] To whom requests for reprints should be addressed, at M. D. Anderson Cancer Center, Department of Head and Neck Surgery, Box 69, 1515 Holcombe Boulevard, Houston, TX 77030.

[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; Ad5CMV-p53, wild-type *p53* adenovirus; CMV, cytomegalovirus; Ad5, adenovirus serotype 5; PFU, plaque-forming units; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

rabbit antihuman *p53* polyclonal antibody, clone OM-1, 1:80 (Signet Laboratories, Denham, MA). An anti-rabbit IgG Elite kit (Vector Laboratories, Burlingame, CA) was then used to apply biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complexes which were incubated for 45 min each. The immunostaining reaction was visualized by using 0.5% dimethylamino-azobenzene in PBS containing 0.01% hydrogen peroxide (pH 7.6), counter-stained with 0.01% toluidine blue, dehydrated, cleared, and mounted in Permount. To verify the specificity of the immunostaining reaction, immunoperoxidase staining was performed (using the same method as on test samples) on a known positive cytospin of a tissue culture of a squamous carcinoma cell line as well as on a negative rabbit monoclonal antibody control.

Inhibition of Tumor Growth *in Vivo*. The effect of Ad5CMV-p53 on a microscopic disease model of SCCHN was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and utilization and the Biosafety Committee for recombinant DNA research. Briefly, nude female mice (aged 4–7 weeks) were anesthetized with i.p. ketamine/acepromazine (70 mg/kg of body weight) (Parke-Davis, Morris Plains, NJ). After their bodies were prepared with alcohol wipes, incisions were made in the dorsal flanks and three s.c. flaps were elevated with sharp dissection. Sterile pipette dispensers were used to introduce the desired number of tumor cells in 100 μl of culture medium into the flap, which was sealed with a horizontal mattress suture. Forty-eight h following tumor-cell delivery, the animals were reanesthetized and the sutures removed. The flap was infected with Ad5CMV-p53, dl312, β-galactosidase adenovirus, or PBS alone (mock infection) by pipetting in 100-μl increments; the flap was then resealed with a horizontal mattress suture. The PFU of the inoculant was increased in log increments with animals serving as their own controls as well as single-flap models on animals. The animals were observed daily for tumor development and killed in cases of excessive tumor burden or after 12 weeks of observation. All surgical sites were evaluated pathologically as well as by necropsy analysis for systemic toxicity.

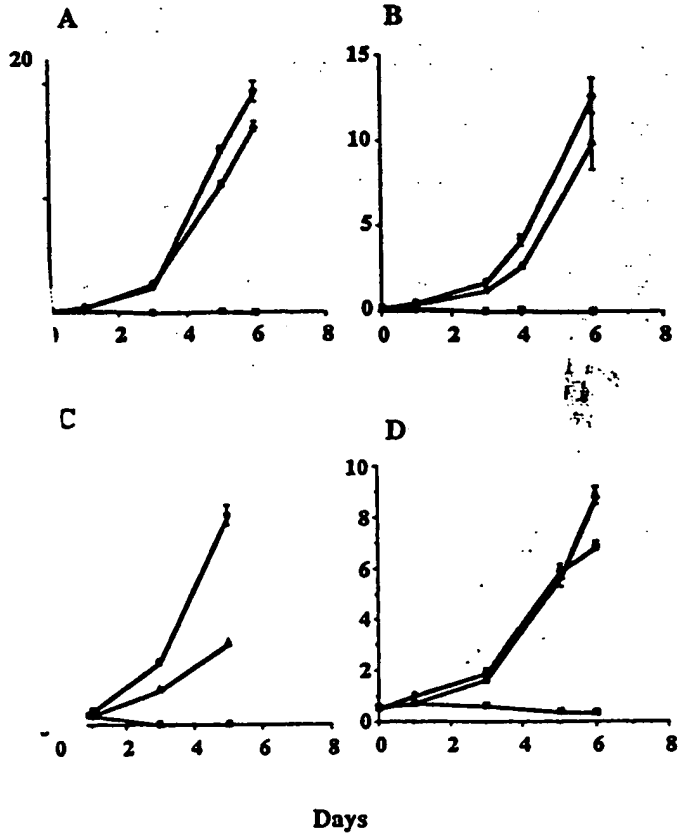
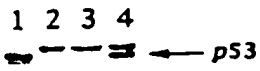


Fig. 1. Composite growth curve of four SCCHN cell lines. *A*, Tu-138; *B*, Tu-177; *C*, MDA 686-LN; *D*, MDA 886. Mock infected cells (●), dl312-infected cells (▲), and Ad5CMV-p53 infected cells (■). The mean of cell counts per triplicate wells following infection were plotted against the number of days post-infection; *bars*, SEM.



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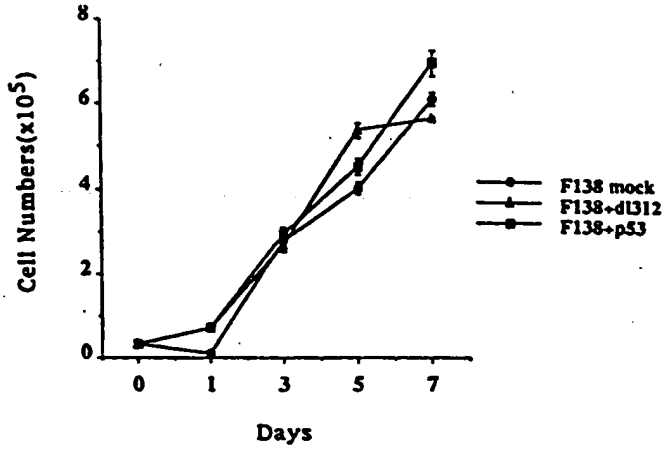

Fig. 2. Expression of exogenous p53 protein in adenovirus infected normal fibroblasts and its effect on growth rate. *A*, Western blot analysis. Cellular extracts isolated from cells 24-h postinfection were subjected to SDS-PAGE. *Lane 1*, Tu-138 infected with the Ad5CMV-p53; *Lane 2*, mock-infection of Fibroblast-138; *Lane 3*, Fibroblast-138 cells infected with the replication-defective adenovirus, dl312; *Lane 4*, Fibroblast-138 cells infected with the Ad5CMV-p53. *B*, Growth curve of normal fibroblast cell line. Mock infected cells (●), dl312 infected cells (▲), and Ad5CMV-p53-infected cells (■).

Results

Effect of Exogenous *p53* on SCCHN Cell Growth *in Vitro*. We described previously the *in vitro* inhibition of cell growth by Ad5CMV-p53 in SCCHN cell lines with endogenously mutated *p53* (8). We therefore sought to determine whether SCCHN cell lines with endogenous wild-type *p53* would be similarly affected. We also investigated the effect of Ad5CMV-p53 on nonmalignant fibroblasts.

Four human SCCHN cell lines were chosen for this study: Tu-138 and Tu-177, which possess a mutated *p53* gene, and MDA 686-LN and MDA 886, which are homozygous for the wild-type *p53* gene.[4] A fibroblast cell line derived from normal fibroblast outgrowth, which is karyotypically normal and nontumorigenic, was used as a nonmalignant control cell line. Cells infected with the control virus, dl312, had growth rates similar to those of the mock-infected cells, whereas the growth of tumor cells infected with Ad5CMV-p53 was significantly suppressed (Fig. 1). Twenty-four-48 h after infection, an apparent morphological change occurred in all tumor cells, with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect occurred earlier in cells with endogenous mutated *p53* than it did in those cells with wild-type *p53*. Cells infected with replication-defective adenovirus, dl312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

[4] G. L. Clayman, unpublished data.

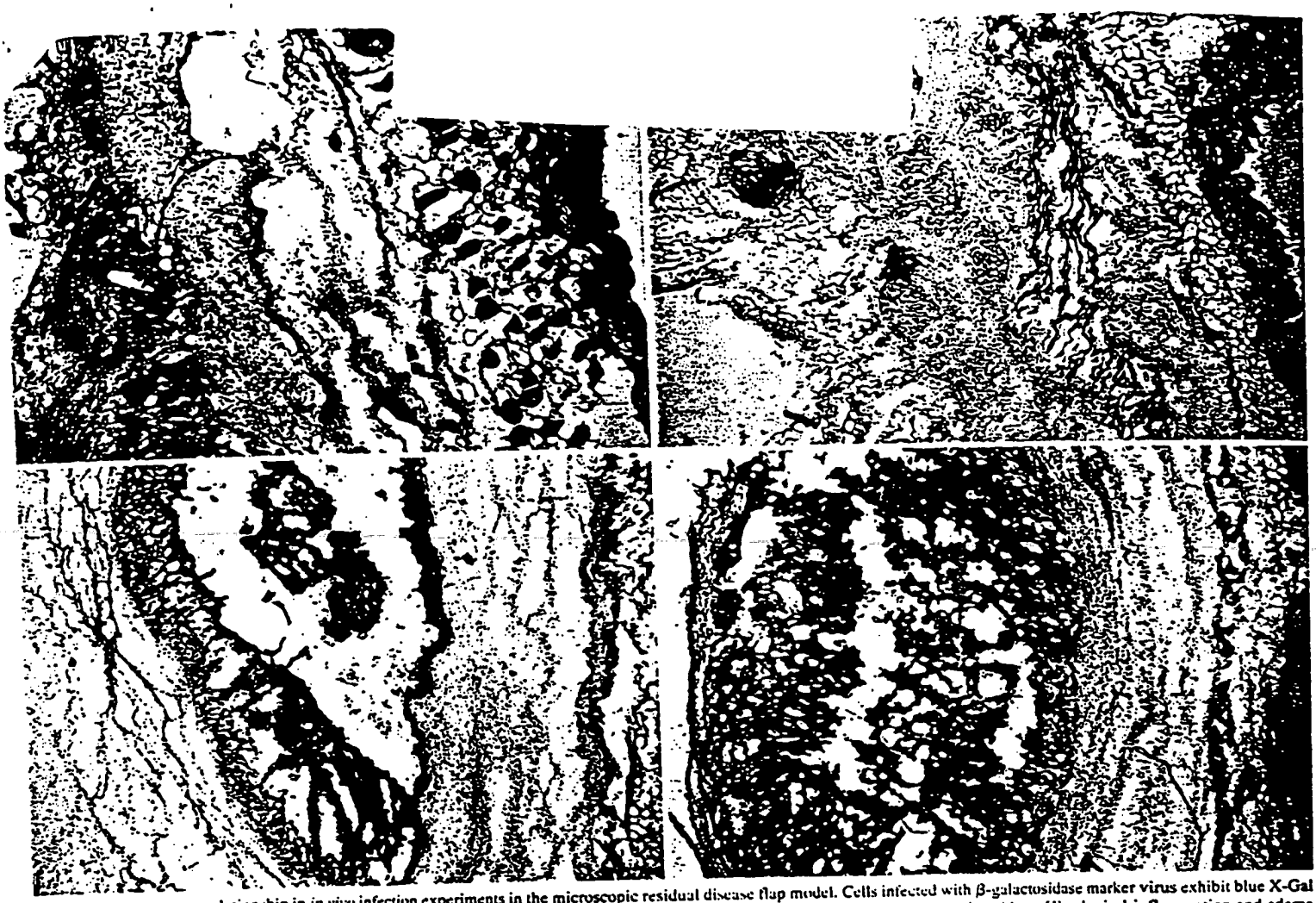

Fig. 3. Dose-response relationship in *in vivo* infection experiments in the microscopic residual disease flap model. Cells infected with β-galactosidase marker virus exhibit blue X-Gal staining. *A*, mock infection: *B*, 10^7 PFU viral particles: *C*, 10^8 PFU viral particles: *D*, 10^9 PFU viral particles. A clear dose response is evident. Histological inflammation and edema increase with increasing viral titers. Magnification, 63x.

Expression of Exogenous p53 Protein in Adenovirus Infected Normal Fibroblasts and its Effect on Growth Rate. Additionally, we investigated the effect of the Ad5CMV-p53 on karyotypically normal and nontumorigenic fibroblast cell lines. These cells were isolated during the establishment of primary tumor cell lines. Twenty-four h after infection. Western blot analysis was performed to compare the levels of protein produced by the different infected cell types. A p53 band, recognized by the monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples infected with the Ad5CMV-p53 (Fig. 2*A*: Lanes 1 and 4). As has been shown previously (8), cell line Tu-138 infected with the *p53* adenovirus showed high levels of p53 protein following transduction and served as a control (Fig. 2*A*: Lane 1). The level of *p53* expression remained similar in both mock-infected and dl312-infected cells (Fig. 2*A*; Lanes 2 and 3). The Ad5CMV-p53-infected fibroblasts showed higher levels of p53 protein than did the control cells (Fig. 2*A*: Lane 4). This result indicates that the *p53* gene is efficiently translated into normal fibroblasts infected with Ad5CMV-p53 as evidenced by production of immunoreactive p53 protein. The protein expression and transduction efficiency of cytospins of Ad5CMV-p53 infected fibroblasts were verified by immunohistochemical analysis (data not shown). This fibroblast cell line exhibited normal growth rate and morphology independent of the intervention (mock, replication-defective virus, or Ad5CMV-p53) (Fig. 2*B*). These experiments were repeated twice and also verified in other normal human fibroblast cell lines.

***In Vivo* Transduction Efficiency.** To measure the efficiency of gene transfer *in vivo*, we resected the s.c. flap site 72 h following molecular or control intervention. Dose-response experiments with the adenovirus β-galactosidase-marker vector demonstrate dose-response transduction efficiency in this model (Fig. 3). This was confirmed with immunohistochemical analysis 4 days following infection with Ad5CMV-p53 (Fig. 4). Both groups of experiments exhibited an *in vivo* dose response which had been described previously *in vitro* by us and others (8). In no instances did doses of virus exceeding 10^{10}-PFU effect expression of *p53* in other organ systems including brain, liver, lung, heart, abdominal visceral organs, and skin (data not shown). These experiments illustrated a dose-response relationship between viral titer and transduction efficiency as well as the possibility of achieving extensive transient expression of the transduced gene within the desired surgical model field.

Suppression of Tumor Growth *in Vivo*. We designed our initial experiments to determine whether *in vivo* Ad5CMV-p53 mediated gene transfer would affect the establishment or growth of SCCHN cells implanted into a s.c. flap. We created a microscopic residual disease model. In this model, three s.c. flaps were elevated on athymic nude female mice, and 2.5×10^5 of tumor cells were seeded by pipetting. Instead of allowing the tumor cells to form nodules

A

B

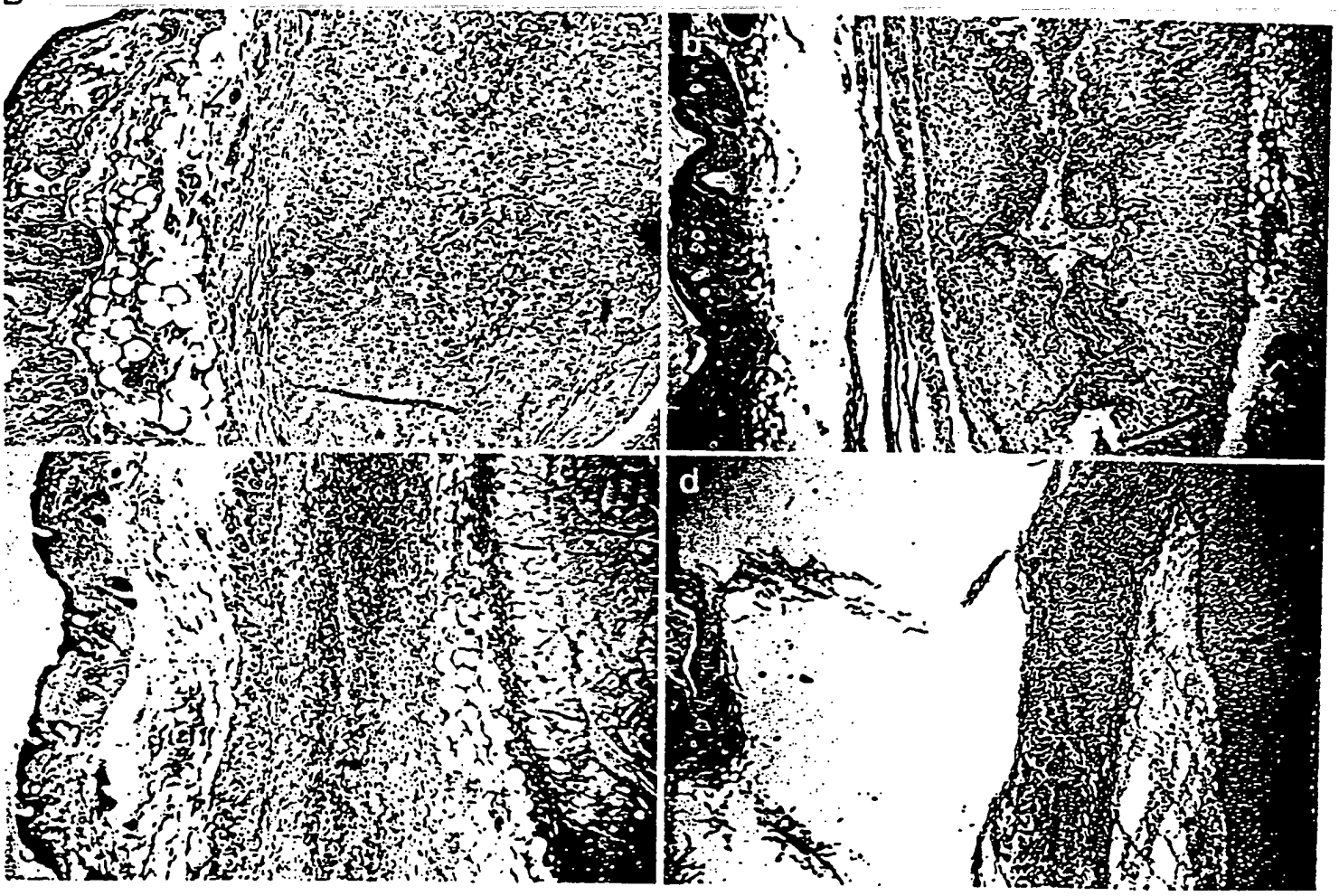

Fig. 4. *A*, Suppression of tumor establishment in a microscopic residual disease model of SCCHN. Nude female mice implanted with Tu-138 cell lines and delivered 48 h later with 100 μl of Ad5CMV-p53 (10^8 PFU) in the right anterior flap dl312 (replication defective adenovirus) 10^8 PFU in the right posterior flap and transport medium (PBS) alone in the left posterior flap. Animals were observed daily and sacrificed at 12 weeks or earlier if excessive tumor burden developed. No gross or histological evidence of tumor in the Ad5CMV-p53 flap site was found. Photograph taken at 3 weeks. *B*, *in vivo* infectivity of Ad5CMV-p53 in the microscopic residual disease flap model. The Ad5CMV-p53 was pipetted into the s.c. flap 48 h following tumor cell line delivery. A representative experiment of the wild-type *p53* SCCHN cell line MDA 886-LN is shown. *Panel a*, mock infection showing lack of immunostaining in the wild-type *p53* cell line; *Panel b*, 10^7 PFU of Ad5CMV-p53; *Panel c*, 10^8 PFU of Ad5CMV-p53; *Panel d*, 10^9 PFU of Ad5CMV-p53. Immunostaining was performed using the polyclonal rabbit antihuman antibody CM1 (Signet Laboratories) using the avidin-biotin method. A clear dose-response relationship is shown. No viable tumor was found in multiple histological sections of 10^8 or 10^9 PFU Ad5CMV-p53. Magnification 100×.

generally occurring in 4 days), we delivered our single dose of molecular intervention at 48 h following tumor cells seeding. In this manner, although no gross tumors were present, microscopic tumor cells were within the surgical site mimicking the clinical dilemma of surgical excision of all gross tumor. The development of tumors was directly related to the number of tumor cells, the time allotted for implantation, and the dose of Ad5CMV-p53. Of the mice which received microscopically implanted tumor cells (2.5×10^6) and were treated with Ad5CMV-p53 at 10^8 PFU or greater, only two mice developed tumors, both of which were implanted with the wild-type *p53* cell line (MDA 886-LN). All other cell lines exhibited absence of tumor development (Table 1). These experiments clearly indicate that the growth of microscopic tumor cells can be effectively suppressed *in vivo* if exposed to the Ad5CMV-p53 (Fig. 4*A*). Tumor formation was evaluated at the end of a 12-week period (earlier animal sacrifice in circumstances of excessive tumor burden) by gross and histological analysis of the surgical sites. The data of tumor establishment is summarized in Table 1, and a representative experiment is shown in Fig. 4*A*.

Immunohistochemical analysis was performed on the tumor sections of experimental animals. A representative experiment of the SCCHN cell line MDA 686-LN is shown (Fig. 4*B*). This cell line possesses the wild-type endogenous *p53* gene. Lack of significant basal immunostaining with the viable tumor of MDA 686-LN (mock-infection) is seen in Panel *A*. Panel *B* (Ad5CMV-p53 at 10^7 PFU) shows peripheral tumor necrosis with immunostaining in the more central portion of the tumor. Panel *C* (Ad5CMV-p53 at 10^8 PFU) reveals total necrosis of the tumor with immunostaining found in the entire surgical pocket with multiple layers expressing protein, including stroma and superficial muscular layers. Panel *D* (Ad5CMV-p53 at 10^9 PFU) shows similar results to that of Panel *C*, however increased exogenous *p53* expression throughout the surgical site and edema are prominent.

Using animals, which served as their own internal controls, implants of 4.0×10^6 or more cells significantly increased the establishment of s.c. implants as compared to the tumor implantation of 2.5×10^6 cells ($P < 0.01$), even when treated at the surgical site with Ad5CMV-p53 48 h after inoculation. Allowing implanted cells to establish for 72 or 96 h prior to the Ad5CMV-p53 intervention similarly increased tumor take (data not shown). Dose-response experiments established that 10^8 and 10^9 PFUs of the Ad5CMV-p53 were equally effective in inhibiting tumor burdens of 2.5×10^6 cells implanted for 48 h (data not shown). Endogenous *p53* status of implanted tumor cell lines (whether homozygous mutated or wild-type *p53*) had little impact on the effectiveness of the Ad5CMV-p53 in the cessation of tumor development.

Table 1 *Effect of Ad5CMV-p53 on tumorigenicity in a microscopic residual disease model of SCCHN*

Forty-eight h post tumor cell implantation, mice were again anesthetized and given a single intervention of either vehicle or viruses [1×10^8 PFU (Ad5CMV-p53 or dl312) each in 0.1 ml] in each of the flap sites. Tumor formation was evaluated at the end of a 12-week period. In further experiments, the wild-type *p53* cell line, MDA 886, exhibited no tumor developed in 5 of 5 animals when the identical treatment strategy with 10^9 PFU Ad5CMV-p53 was delivered at 48 h.

	Treatment No. of mice developing tumors/total mice Vehicle		
Cell line	PBS	dl312	Ad5CMV-p53
Tu-138 (homozygous mutation *p53*)	8/8	8/8	0/8
Tu-177 (homozygous mutation *p53*)	8/8	8/8	0/8
686-LN (homozygous wild-type *p53*)	5/8	5/8	0/8
886 (homozygous wild-type *p53*)	6/6	6/6	2/6

Discussion

Tumor suppressor genes are only one of several groups of genes whose transfer might be useful for the local and regional treatment of cancer. Increasing immune surveillance by enhancing of MHC antigen expression or inducing local cytokines to activate the immune system can induce a local tumor effect. Another approach is introducing a gene that may induce apoptosis or that would make a tumor sensitive to particular chemotherapeutic agents. Furthermore, focusing on cell-cycle arrest in malignancies may prove beneficial.

At least two gene "suicide vectors" have already been described; the herpes simplex thymidine kinase gene allows infected cells to be treated with ganciclovir while noninfected cells are unaffected, and the bacterial enzyme cytosine deaminase gene allows infected cells to convert 6'-fluorocytosine to 5-fluorouracil. Unlike these agents, Ad5CMV-p53 has shown no significant toxic effect on untransformed cells and its expression is transient, lasting about 15 days (data not shown).

In vitro experiments have shown that Ad5CMV-p53 stops cell growth regardless of the endogenous *p53* gene status of the tumor cells. The mechanism by which abundant overexpression of wild-type *p53* protein induces this effect appears to be apoptosis, but this requires further investigation. It is important that normal fibroblasts transduced by this vector express the wild-type protein at levels similar to the SCCHN cell lines but without inhibition of cell growth or abnormalities in morphology. The unique molecular events occurring within these transformed tumor cell lines that dispose them to cell death following Ad5CMV-p53 transduction (while nonmalignant cells are spared) requires elucidation. Nevertheless, this sparing of normal cells further supports the potential for molecular therapy with this vector, since transduction of normal cells will be unavoidable in *in vivo* human trials.

Clearly a pure viral effect was also noted on delivery to the s.c. pockets, however this did not appear to be tumoricidal in these experiments. No clinical or histological findings of inflammation or edema different from those at the control sites were noted when the mice were treated with replication-defective virus or adenovirus β-galactosidase at 10^7 PFU or less. At 10^8 PFU and higher doses, however, inflammatory polymorphonuclear leukocytes and edema were histopathologically evident in the pocket sites, although no soft-tissue compromise was clinically evident. These conditions were not seen in mice mock-infected with transport medium alone or with lower viral doses.

In vitro studies in our laboratory have shown approximately 70% adenovirus transduction among SCCHN cell lines in a single exposure under optimal conditions. Nevertheless, the *in vivo* studies clearly showed significant suppression of tumor development from tumor burdens of 2.5×10^6 cells. Whether this reflects a need for a particular minimum tumor burden for tumors to develop, a "bystander" infectious environment, or an insensitivity of our assays to determine the effectiveness of molecular transfer has not been established.

These studies were designed to evaluate the efficacy and potential therapeutic role of *in vivo* gene transfer in a local-regional microscopic residual disease model. They revealed an effective *in vivo* mechanism for molecular therapy in head and neck cancer and suggest that this model delivery system may have a profound impact in the management of microscopic residual disease.

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Apoptosis Induction Mediated by Wild-Type *p53* Adenoviral Gene Transfer in Squamous Cell Carcinoma of the Head and Neck[1]

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ABSTRACT

Cancer gene therapy strategies for inducing apoptosis in solid tumors may allow contemporary medicine to reassess its management of these cancers. We demonstrated previously that overexpression of the wild-type *p53* gene in squamous cell carcinoma of the head and neck cell lines via adenovirus-mediated gene transfer suppressed growth both *in vitro* and *in vivo*. Here, we characterize the mechanism of the growth suppression by the exogenous *p53* gene as a consequence of programmed cell death (apoptosis). One of the cell lines used in this study, Tu-138, harbors a mutated *p53* gene, whereas the other cell line, MDA 686LN, possesses a wild-type *p53* gene. DNA fragmentation was detected by electrophoresis in both cell lines after infection with the wild-type *p53* adenovirus, Ad5CMV-*p53*. With the use of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling method, 4.4% of the remaining viable Tu-138 cell population was identified as apoptotic as early as 15 h after inoculation with Ad5CMV-*p53*. The percentage of apoptotic cells increased to 31% at 22 h. In contrast, only 10% of the viable MDA 686LN cells (wt-*p53*) had undergone apoptosis 30 h after Ad5CMV-*p53* infection, although the percentage of apoptotic cells rapidly increased to 60% at 48 h after infection. For *in vivo* analysis of apoptosis, nude mice in which squamous cell carcinoma of the head and neck cell lines had been implanted s.c. had exogenous wt-*p53* transiently introduced to the tumor cells via Ad5CMV-*p53* 2 days later. *In situ* end labeling clearly illustrated apoptosis in the tumor cells. These results suggest that wt-*p53* plays an important role in the induction of apoptosis in human head and neck cancer cell lines and that selective induction of apoptosis in cancer cells can be further explored as a strategy for cancer gene therapy.

INTRODUCTION

Balancing the rates of cell proliferation and cell death is important in maintaining normal tissue homeostasis. Disruption of this balance may be a major factor in the multistep process of tumorigenesis, and inhibition of apoptosis, or programmed cell death, is one cause of this disruption. Apoptosis also occurs during normal embryogenesis, in the course of normal tissue turnover, after withdrawal of a trophic hormone from its target tissue, and in thymic regression, offering excellent opportunities to study the apoptotic process. We contend that the most promising new therapies for solid malignancies are interventions at the molecular level, and that selective induction of apoptosis in these cancers is a logical intervention strategy. The gene or genes that may induce cancer cell apoptosis continue to be investigated, as do the methods for gene transfer. Presently, adenovirus-mediated gene transfer is our clinical method of choice for such interventions because of its known tropism for the epithelium of the aerodigestive tract, its excellent transduction efficiency, the transient nature of gene expression in the cells it infects (lack of permanent integration), and its ability to infect nonproliferating cells. The *p53* gene also continues to be of interest as a molecular therapy for some solid malignancies.

p53 was originally discovered through its association with the SV40 large T antigen (1, 2). The importance of the *p53* gene product in human neoplasia was first recognized a few years ago when mutant forms of the gene were identified in human colorectal tumors. Subsequently, *p53* mutations have been identified in the majority of human malignant solid tumors, including those of the breast (3), colon (4), lung (5), and oral cavity (6). Several studies have demonstrated the ability of the wt-*p53*[3] to suppress cancer cell growth both *in vitro* and *in vivo*, suggesting that it acts as a tumor suppressor gene. The suppression of cell growth by *p53* is mediated by two distinct pathways, one transient and one permanent. In the case of transient suppression, *p53* serves as a cell cycle checkpoint regulator. Overexpression of wt-*p53* has been shown to induce a reversible cell cycle arrest at the G_1-S boundary (7, 8). In other instances, *p53* may induce apoptosis when overexpressed in some cultured cells (9, 10) and is required for DNA damage-induced apoptosis in mouse thymocytes (11).

We reported previously that overexpression of the wt-*p53* in SCCHN cell lines induced via adenovirus-mediated gene transfer suppressed growth both *in vitro* and *in vivo* (12, 13). Here, we sought to examine the mechanism of this growth suppression. Our data indicate that the suppression effect, both *in vitro* and *in vivo*, is the consequence of an irreversible event, apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: Recombinant Adenovirus Preparation and Infection. Human SCCHN cell lines Tu-138 and MDA 686LN were all established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, and had been characterized previously (12, 13). All procedures were performed and cell lines maintained as described previously (12, 13). Cell growth assays were performed in triplicate.

DNA Fragmentation Analysis. After incubation with Ad5CMV-*p53* or replication-defective adenoviral controls at various time intervals, cells were harvested and resuspended in 300 μl of PBS, to which 3 ml of extraction buffer [10 mM Tris (pH 8.0)-0.1 M EDTA-20 μg/ml RNAse-0.5% SDS] were added before incubation at 37°C for 1–2 h. At the end of incubation, proteinase K was added to a final concentration of 100 μg/ml, and the solution was placed in a 50°C water bath for at least 3 h. DNA was extracted once with an equal volume of 0.5 M Tris (pH 8.0)-saturated phenol and then again with phenol/chloroform. Precipitated DNA was analyzed in a 1% agarose gel.

Cell Fixation. Before the TUNEL method was used to identify apoptotic cells, the cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 30 min on ice. Cells were then washed with 3 ml of PBS, resuspended in 70% ice-cold ethanol, and stored at −20°C until used. For cell cycle analysis, cells were fixed in 70% ice-cold ethanol only.

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[3] The abbreviations used are: wt-*p53*, wild-type *p53*; SCCHN, squamous cell carcinoma of the head and neck; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Ad5CMV-*p53*, wild-type *p53* adenovirus; Tdt, terminal deoxynucleotidyl transferase.

Terminal Deoxynucleotidyl Transferase Assay. The TUNEL assay was performed according to the procedure of Gorczyca *et al.* (14). Briefly, after fixation and washing, cells were resuspended in 50 μl of TdT buffer containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM Tris-HCl, 2.5 mM $COCl_2$ (Sigma Chemical Co., St. Louis, MO), 0.1 mM DTT (Sigma Chemical Co.), 0.25 mg/ml bovine serum albumin (Sigma Chemical Co.), 5 units of terminal transferase (Boehringer Mannheim, Indianapolis, IN), and 0.5 nmol biotin-16-dUTP along with dATP, dGTP, and dCTP at concentrations of 20 μM. Controls were prepared by incubating a separate aliquot of each test sample without dUTP. The cells were incubated in the solution at 37°C for 30 min, rinsed in PBS, and resuspended in 100 μl of fluorescein isothiocyanate, the staining solution containing 4X SSC, 0.1% Triton X-100, and 2.5 μg/ml fluoresceinated avidin (Vector Laboratories, Inc., Burlingame, CA). Tubes were incubated for 30 min in the dark at room temperature. Cells were rinsed in PBS with 0.1% Triton X-100 and resuspended in 0.5 ml PBS containing propidium iodide (5 μg/ml) and 70 μl (1 mg/ml) RNAse. Tubes were incubated in the dark on ice for 30 min before flow cytometric analysis.

Flow Cytometric Analysis. All samples were analyzed with the use of an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) with the standard optical configuration. At least 10,000 events were counted for each sample. Positivity for TdT end labeling was determined by subtracting the control histogram from the test histogram with the use of the Immuno-4 program of the Elite workstation software (Coulter Corp.).

Cell Growth Assay. Cells were plated at a density of 2×10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either the Ad5CMV-*p53* or the replication-defective adenovirus (dl312) as a control. Cells were harvested at different time intervals and counted, and their viability was determined by trypan blue exclusion.

***In Vivo* Analysis for Apoptosis.** Gene therapy in a microscopic residual disease model of SCCHN has been described elsewhere (13). Experiments were reviewed and approved by the institutional committees for both animal care and utilization and the Biosafety Committee for recombinant DNA research. Briefly, s.c. flaps were elevated in anesthetized 4–6-week-old nude female mice with sharp dissection, and 2.5×10^6 tumor cells in 100 μl of culture medium were pipetted into the flap and sealed with a horizontal mattress suture. Forty-eight h after tumor cell delivery, animals were reanesthetized and infected with Ad5CMV-*p53*, replication-defective virus (dl312), or PBS alone (mock infection). The animals were observed daily and killed 72 h after the second, or "therapeutic," intervention.

***In Situ* End Labeling.** The procedure was performed as described elsewhere (15). Briefly, paraffin-embedded tumor sections were dewaxed in xylene for 5 min three times each and were progressively hydrated by immersing the slides for 3 min each in 100, 90, 70, and 30% ethanol solutions. Endogenous peroxidase was inactivated by immersing the slides for 20 min in 0.75% H_2O_2 (v/v) in 100% methanol. After the slides were washed in PBS, sections were digested with 0.1% pepsin (Fisher Scientific, Houston, TX; w/v) in 0.1 N HCl for 5 min at 37°C and extensively washed in PBS. Sections were then incubated in a moist chamber at 37°C for 1 h with an end-labeling cocktail that included the following: 0.5 unit/μl TdT; 0.06 mM biotinylated dUTP; 10 μl 5× TdT buffer; and double-distilled water up to 50 μl. The reaction was terminated by immersing the slides in a buffer containing 300 mM NaCl and 30 mM sodium citrate in double-distilled water. After the slides were washed in PBS, sections were incubated with horseradish peroxide-conjugated avidin for 1 h at 37°C in a moist chamber. Staining was developed using 3,3'-diaminobenzidine, and sections were counterstained with methyl green.

RESULTS

Suppression of SCCHN Cell Line Growth by Ad5CMV-*p53*. We reported previously that wt-*p53* can be efficiently transduced into SCCHN cell lines by a recombinant adenoviral vector. Consequently, the insulted tumor cells lose their ability to proliferate *in vitro* as well as *in vivo*. The suppression effect is independent of the endogenous *p53* status of the cell lines. Previous growth rate analyses were carried out through a 1-week period. Here, we sought to investigate the early effects of the wt-*p53* on SCCHN cell growth (*i.e.*, after shorter time intervals).

Two representative cell lines were used in this study. Cell line Tu-138 harbors a mutated *p53* gene, whereas cell line MDA 686LN possesses a wt-*p53* gene. Cells infected with the replication-defective virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 1, *A* and *B*). On the other hand, growth of the Ad5CMV-*p53*-infected Tu-138 (Fig. 1*A*) and MDA 686LN (Fig. 1*B*) cells was significantly suppressed. It appeared that the exogenous p53 protein had an earlier and more profound suppressive effect on Tu-138 than on MDA 686LN. An apparent morphological change was observed in both cell lines, with portions of the cell populations rounding up and their outer membranes forming blebs. This resembled apoptosis and occurred concomitantly with the initiation of the growth suppression. Cells infected with the replication-defective adenovirus dl312 demonstrated normal growth characteristics with no histomorphological abnormalities. It is important that these effects were not observed after infection by Ad5CMV-*p53* of karyotypically normal fibroblasts (13) or human oral keratinocytes (immortalized but nontumorigenic with endogenous wt-*p53*).[4]

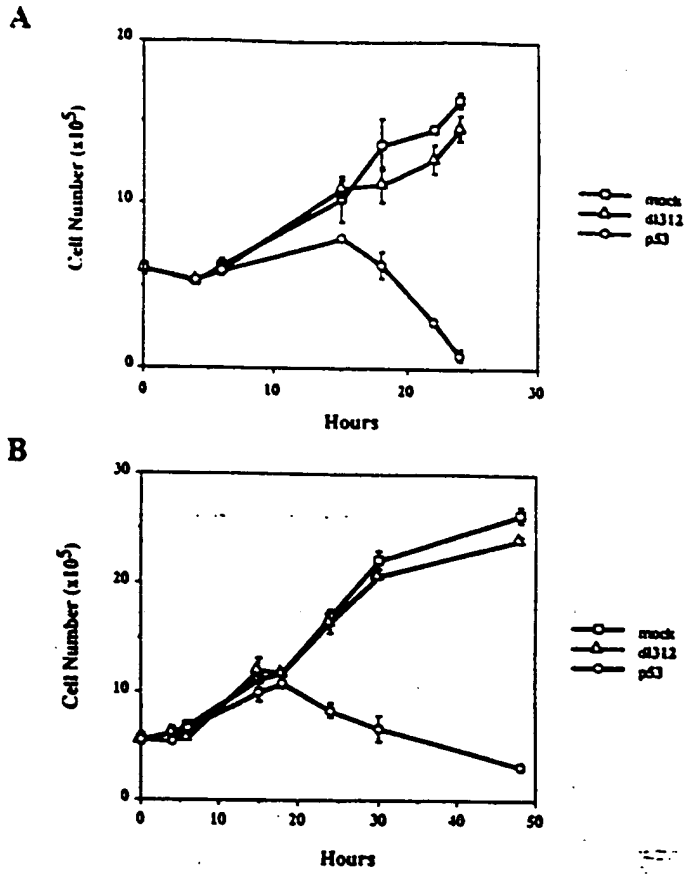

Fig. 1. Composite growth curve of SCCHN cell lines. *A*, Tu-138; *B*, MDA 686LN. At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate dishes were plotted against the number of hours postinfection. *Bars*, SEM.

DNA Fragmentation Analysis. One of the characteristic markers of apoptosis that distinguishes it from necrosis is the biochemically observable appearance of the ladder of DNA fragments. To confirm the assumption that the cells had undergone apoptosis after the Ad5CMV-*p53* infection, we performed DNA fragmentation analysis. Chromosomal DNA extracted from the viable cells after infection with the replication-defective virus or Ad5CMV-*p53* was subjected to agarose gel electrophoresis. The appearance of DNA fragments equivalent to approximately 200 bp, and their multiples

[4] G. L. Clayman, unpublished data.

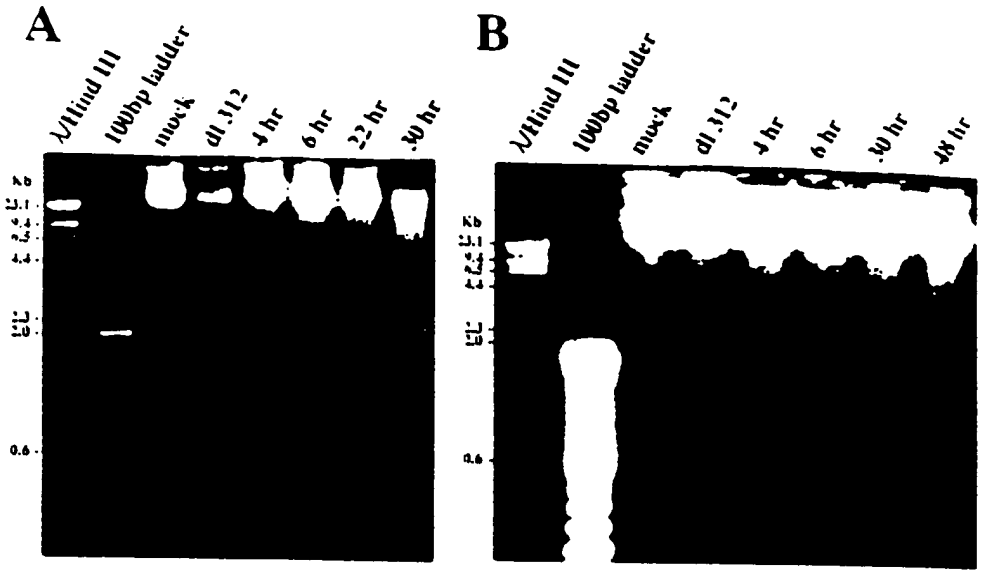

Fig. 2. *In vitro* DNA fragmentation analysis after infection with Ad5CMV-*p53*, mock virus, or replication-defective virus. *A*, Tu-138 cells (endogenous mutated *p53*); *B*, MDA 686LN cells (endogenous wt-*p53*). Both SCCHN cell lines were analyzed in a time course experiment. *Lanes 1* and *2* (from *left*), λ *Hin*dIII and 100-bp DNA ladders served as molecular markers, respectively; *Lane 3*, mock infection at 24 h; *Lane 4*, replication-defective virus infection at 24 h; *Lanes 5* and *6*, Ad5CMV-*p53* infection at 4 and 6 h, respectively; *Lanes 7* and *8*, Ad5CMV-*p53* infection at time periods as shown.

was noticed in both cell lines (Fig. 2). The fragmented DNA appeared 22 h after Ad5CMV-*p53* infection in the Tu-138 cell line (Fig. 2*A*) and at 30 h in the MDA 686LN cell line (Fig. 2*B*), although they were more evident in the latter at 48 h. No detectable fragmented DNA emerged from the mock-infected or dl312-infected cells.

***In Vitro* TdT Assay.** Another characteristic marker of apoptosis is the morphological change and destruction of the structural organization of the nucleus, which results in chromatin condensation. Electron microscopy has been used extensively to detect such ultrastructural alteration. Recently, however, flow cytometric methods for identifying apoptotic cells have gained favor because of their greater ability to scan and analyze cellular populations (14). The TUNEL method used here is based on detection of the extensive DNA breakage to identify the apoptotic cells. Fifteen h after infection with Ad5CMV-*p53*, 4.4% of the viable Tu-138 cell population was in apoptotic stages (Fig. 3*A*), yet we were unable to detect any fragmented DNA in their agarose gels done at that time.[4] There appeared to be a delay in apoptosis induction in the MDA 686LN cells, with 4% of cells exhibiting detectable apoptosis 18 h after the infection with Ad5CMV-*p53* (Fig. 3*B*). This apparent discrepancy between the TUNEL assay and fragmentation analysis is most likely due to the high sensitivity of the TUNEL assay. The number of apoptotic cells increased proportionally to the duration of the Ad5CMV-*p53* incubation. Nearly 31% of the Tu-138 cells had undergone apoptosis at 22 h. Although induction of apoptosis was at first delayed in MDA 686LN cells, approximately 60% of these cells were in apoptotic stages 48 h after Ad5CMV-*p53* infection. It is noteworthy that the percentage of apoptotic cells may have been significantly underestimated by the TUNEL method because only viable cells were subjected to the analysis. These data correlated well with the growth rate and DNA fragmentation analyses. There was no detectable apoptosis in control experiments with the use of mock infection or replication-defective viral controls (100 multiplicity of infection). Therefore, apoptosis did not appear to be a function of the transduced adenoviral gene products themselves. Subsequently, cells were counterstained with propidium iodide for their total DNA content and visualized with the use of nuclear fluorescence (Fig. 4, *A–D*). Fig. 4, *A* and *B* (Tu-138) and *C* and *D* (MDA 686LN), reveals that cells exhibit orange nuclear fluorescence with normal nuclear anatomy after infection with replication-defective virus (Fig. 4, *A* and *C*). In contrast, cells infected with Ad5CMV-*p53* showed green fluorescence with nuclear fragmentation (Fig. 4, *B* and *D*).

***In Vivo* Analysis for Apoptosis.** We showed previously that Ad5CMV-*p53* could suppress tumor formation *in vivo*. In this study, we investigated whether this suppression was the consequence of apoptosis. *In situ* end-labeling analysis was performed to detect apoptotic cells in paraffin-embedded sections obtained from our previous study (13, 15). No staining was observed in the tissue sections isolated from MDA 686LN-bearing animals which had received PBS treatment only as controls (Fig. 4*E*). On the other hand, tissue sections isolated from MDA 686LN-bearing mice treated with Ad5CMV-*p53* were highly stained (Fig. 4*F*), suggesting that apoptosis was the event involved in suppression of tumor growth *in vivo*.

It has been shown that the *WAF1/CIP1* gene, which may be induced after wt-*p53* overexpression, is involved in cell cycle arrest (16). We sought to determine whether the tumor growth suppression we saw was due in part to the cell cycle arrest by the induced p21 protein or primarily to apoptosis. Western blotting showed that the p21 protein was induced in the Ad5CMV-*p53*-infected SCCHN cells. However, cell cycle analyses indicated that despite the elevated level of p21 protein in the Ad5CMV-*p53*-infected cells, there was no significant accumulation of cells at G_1 as compared to S phase.[4]

DISCUSSION

Tumor suppressor genes are only one of several groups of genes the transfer of which might be useful for the local and regional treatment of solid malignancies. Certainly, because of considerable evidence implicating mutations of the *p53* gene in human cancers, this has been one of the most extensively studied tumor suppressor genes to date. Studies have demonstrated that the growth of several different human cancer cell lines including colon (9), breast (17), osteosarcoma (18), and non-small cell lung cancer (19), can be functionally suppressed by wt-*p53* with the use of a variety of methods of gene transfer. The mechanism through which wt-*p53* expression mediates its control, however, requires further elucidation.

wt-*p53* has been shown to be involved in several aspects of cell growth control (20, 21). One of the functions of wt-*p53* as a tumor suppressor gene is to induce apoptosis in damaged cells. This process, however, is dependent on several known but yet to be characterized factors, not to mention cell type of origin and induced downstream effectors (9, 10).

We demonstrated previously that the introduction of wt-*p53* into SCCHN cell lines by a recombinant adenoviral vector suppressed the

growth of tumors both *in vitro* and *in vivo*. In the study presented here, we sought to determine the mechanism responsible for the irreversible cytocidal effect of the recombinant wt-*p53* adenovirus in SCCHN.

Because transfer and overexpression of wt-*p53* initiated tumor growth suppression, and the wt-p53 protein is one of several gene products that may induce apoptosis, we investigated whether apoptosis was the mechanism involved in SCCHN. *In vitro* growth analysis showed that the growth of both SCCHN cell lines was suppressed by the exogenous expression of wt-p53 protein, independent of their endogenous *p53* status (whether mutated or wt). The effect occurred earlier and more profoundly in the endogenously mutated cell line, but growth was totally suppressed within 72 h in both of the SCCHN cell lines used (13). Whether this delay in suppression was a function of the endogenous p53 status of that cell line or of other related or unrelated molecular events remains to be investigated. It is important that karyotypically normal human fibroblasts (with endogenous wt-*p53*) were insensitive to Ad5CMV-*p53* treatment despite adequate transduction. Similarly, nonmalignant human immortalized oral keratinocytes were equally unaffected by such treatment. These studies illustrate the specificity of this molecular approach, as well as its sparing of normal tissues, even if they are transduced.

The SCCHN cell lines infected with Ad5CMV-*p53* clearly manifested apoptosis. Nucleosomal DNA analysis revealed characteristic fragmented DNA laddering in both cell lines, with fragmentation occurring earlier in the endogenously mutated cells. Replication-defective virus produced no effect, suggesting that the process was not a function of transduced viral gene products unrelated to the *p53* gene. *In vitro* TdT analysis corroborated both growth rate and DNA fragmentation analyses, although the assay proved to be more sensitive for the detection of earlier nuclear fragmentation and allowed the perusal of the overall tumor cell population. Moreover, *in situ* end labeling illustrated that apoptosis was also the mechanism involved in suppression of tumor growth *in vivo*.

Multiple pathways may exist for induction of apoptosis, and these pathways may be regulated by interventions from upstream and/or downstream factors. Accumulating evidence suggests that both *p53*-dependent and *p53*-independent apoptotic pathways exist (11, 22, 23). It is likely that multiple factors determine whether *p53* mediates cell cycle arrest or apoptosis, including the histological source and embryonic origin of the cells, the constitutive molecular make-up of the cells, and factors relating to the transformation process. *p53* is probably not a requirement for apoptosis in embryonic development because "*p53* knock-out" mice develop normally (24). In contrast, *p53* may be extremely important as a critical defense mechanism to induce apoptosis in cells that overexpress oncogenes or mutated tumor suppressor genes or are virally transformed. This has been shown in a Burkitt's lymphoma cell line which constitutively expressed an active c-*myc* oncogene and underwent apoptosis after expression of exogenous *p53* (25). Additionally, other factors, including the *Bcl* family of genes and adenoviral E1B proteins, may block apoptosis in both *p53*-dependent and *p53*-independent pathways (26).

The induction of *WAF1/CIP1* in *p53*-mediated G_1 arrest and apoptosis in response to DNA damage has also been reported (16). In our

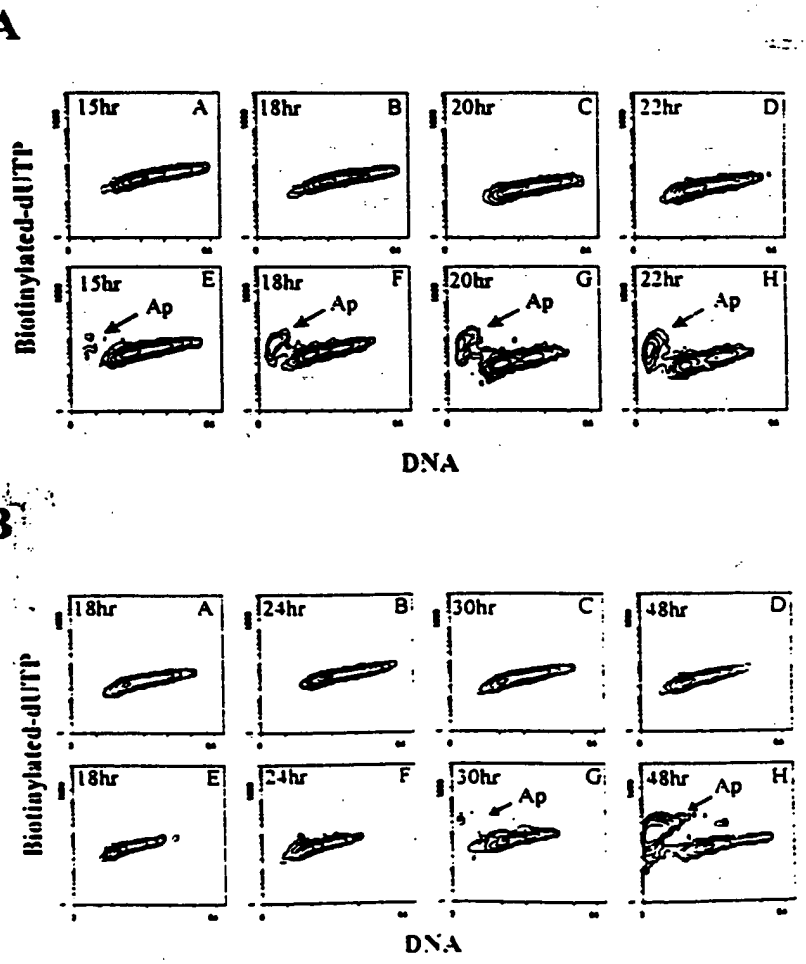

Fig. 3. Labeling of DNA breaks in apoptotic cells with biotinylated dUTP by TUNEL method. After infection, flow cytometric analysis for apoptosis was performed in a time course experiment. *A*, Tu-138 cells which are infected with dl312, a replication-defective adenovirus (*A–D*), or Ad5CMV-*p53* (*E–H*). *B*, MDA 686LN cells which are infected with dl312, a replication-defective adenovirus (*A–D*), or with Ad5CMV-*p53* (*E–H*). *Ap*, apoptosis.

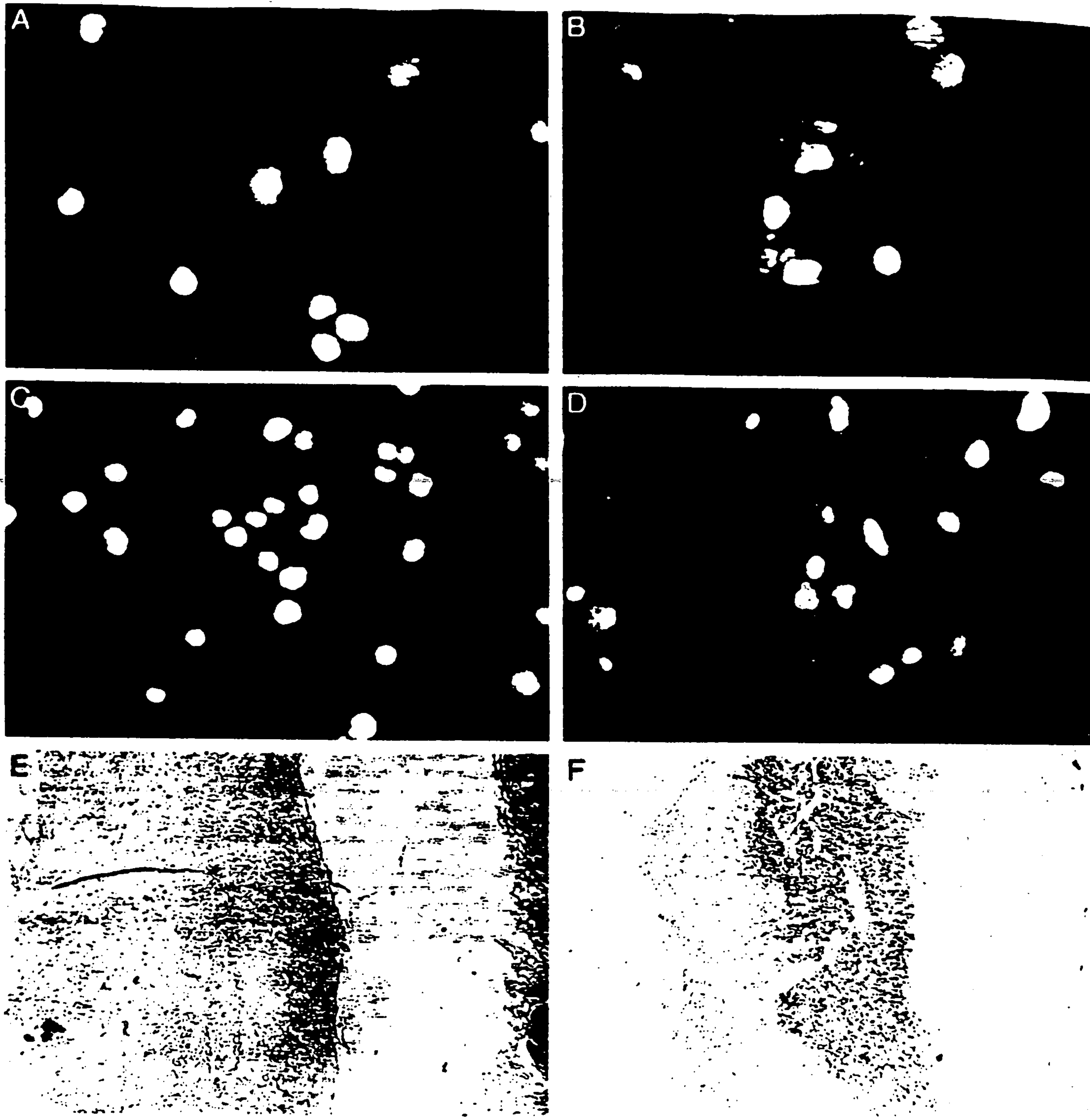

Fig. 4. [illegible] analysis for apoptosis by nuclear fluorescence staining of SCCHN cell lines Tu-138 cells (A, B) and MDA 686LN cells (C, D). [illegible] after infection cells were [illegible] and fixed in [illegible] formaldehyde. The TUNEL reaction was performed as described in "Materials and Methods." [illegible] were then counterstained with propidium iodide to visualize the total DNA content. A and C, orange staining of both cell lines infected with replication-defective virus [illegible] multiplicity of infection [illegible] B and D [illegible] of green [illegible] apoptosis and orange fluorescence in both cell lines infected with Ad5CMV-p53 adenovirus [illegible] multiplicity of infection. Nuclear fragmentation [illegible] seen in the cells infected with Ad5CMV-p53 [illegible]. E and F, [illegible] analysis for apoptosis in an animal model of microscopic residual disease [illegible] performed in paraffin-embedded sections obtained from MDA 686LN tumor-bearing [illegible] from our previous study [illegible] PBS [illegible] significant staining for apoptosis. The other tumor was treated with [illegible] forming units of Ad5CMV-p53, which suppressed [illegible] growth and caused extensive apoptosis [illegible] tumor (F). Apoptosis was not visualized in any surrounding normal tissues [illegible]

study, despite the induction of WAF1/CIP1 protein expression in the Ad5CMV-p53-infected cells,[illegible] cell cycle analysis revealed no evidence of arrest at G[illegible] before apoptosis, indicating that growth arrest is not a prerequisite for apoptosis but is a distinct process. Recently, E2F-[illegible], a transcription factor that forms a complex with the retinoblastoma susceptibility gene product, *RB*, has been shown to cooperate with *p53* in mediating apoptosis (27), indicating that the two tumor suppressor gene products *RB* and *p53* interact with each other as checkpoint control regulators of the cell cycle. The molecular mechanisms that mediate such communication, as well as cross-talk among

other important cell cycle-regulating components such as *p16*, *p21*, cyclins, and cyclin-dependent kinases remain to be elucidated.

In summary, the studies presented here demonstrated that Ad5CMV-*p53* induced apoptosis in SCCHN but spared normal cells. In contrast, Roth *et al.* (19) found that the same intervention suppressed the growth of malignant non-small cell lung cancer cells (H358) but did not induce apoptosis. This supports the concept that there are inherent constitutive differences between these neoplasms that may regulate the apoptotic process. We conclude that selective induction of apoptosis of solid malignancies that spares normal cells is an attractive strategy for molecular therapy and requires further investigation.

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